Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in *Smad4/Dpc4* conditional knockout mice

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Summary

Smad4 is a central mediator for TGFβ signals, which play important functions in many biological processes. To study the role of Smad4 in mammary gland development and neoplasia, we disrupted this gene in mammary epithelium using a Cre-loxP approach. *Smad4* is expressed in the mammary gland throughout development; however, its inactivation did not cause abnormal development of the gland during the first three pregnancies. Instead, lack of Smad4 gradually induced cell proliferation, alveolar hyperplasia and transdifferentiation of mammary epithelial cells into squamous epithelial cells. Consequently, all mutant mice developed squamous cell carcinoma and/or mammary abscesses between 5 and 16 months of age. We demonstrated that absence of Smad4 resulted in β-catenin accumulation at onset and throughout the process of transdifferentiation, implicating β-catenin, a key component of the Wnt signaling pathway, in the development of squamous metaplasia in Smad4-null mammary glands. We further demonstrated that TGFβ1 treatment degraded β-catenin and induced epithelial-mesenchymal transformation in cultured mammary epithelial cells. However, such actions were blocked in the absence of Smad4. These findings indicate that TGFβ/Smad4 signals play a role in cell fate maintenance during mammary gland development and neoplasia.

Key words: Smad4/Dpc4, TGFβ, Transdifferentiation, Keratinocytes, Neoplasia

Introduction

Mammary gland development starts during the embryonic stages but most ductal growth and branching morphogenesis take place during puberty. With each pregnancy, mammary tissue undergoes a programmed cycle of epithelial cell proliferation, lobuloalveolar differentiation, lactation and involution. Although this developmental cycle is dependent on systemic steroids and peptide hormones, it is clear that additional levels of control and regulation must be involved. In experimental animal systems, alterations of many genes have been shown to cause abnormalities in mammary gland development and tumorigenesis (Cardiff et al., 2000; Deng and Brodie, 2001; Hennighausen and Robinson, 2001). Specifically, both gain- and loss-of-function experiments have implicated transforming growth factor beta (TGFβ) signals in both mammary development (Buggiano et al., 2001; Gorska et al., 1998; Joseph et al., 1999; Nguyen and Pollard, 2000) and tumor formation (reviewed by Akhurst, 2002; Derynck et al., 2001; Wakefield et al., 2001; Wakefield and Roberts, 2002).

Mammalian TGFβs constitute a superfamily of over 40 secreted signaling molecules, which function in diverse developmental processes by regulating proliferation, differentiation and apoptosis (reviewed by Derynck et al., 2001; Wakefield et al., 2001; Wakefield and Roberts, 2002). Members of a TGFβ subgroup (TGFβ1-TGFβ3) and their receptors are expressed in the mammary epithelium and terminal endbuds during branching morphogenesis and have been identified as important regulators of mammary epithelial cell proliferation, differentiation and transformation (Buggiano et al., 2001; Gorska et al., 1998; Joseph et al., 1999; Nguyen and Pollard, 2000).

TGFβ signals have been implicated in breast cancer formation. Breast cancer cell lines, primary breast cancers and invasive carcinomas show increased expression of TGFβ1 (Chakravarthy et al., 1999). Consistently, TGFβ1 was also found to induce both estrogen-dependent and -independent tumorigenicity of human breast cancer cells in nude mice (Arteaga et al., 1993), whereas inhibition of TGFβ signals inhibits breast cancer cell tumorigenicity (Muraoka et al., 2002; Yang et al., 2002b). However, ectopic expression of TGFβ1 in transgenic mice represses 7, 12-dimethylbenz[a]anthracene-induced mammary tumor formation (Pierce et al., 1995). The decreased incidence of mammary tumor was correlated with the inhibition of TGFβ on the proliferative activity of mammary epithelial cells and mammary stem cells (Boulanger and Smith, 2001).

Consistently, a dominant-negative form of TGFβ type II receptor (TGFβ-DNIIR), which blocks TGFβ responsiveness, has been found to cause mammary tumor formation in response to carcinogen (Bottinger et al., 1997a). Therefore, it was proposed that TGFβ has biphasic actions on tumors cells, i.e. it is an important negative growth effector at an early stage, but later enhances the malignant conversion and invasion, primarily through the induction of epithelial-mesenchymal transformation (EMT) (Oft et al., 2002; Piek et al., 1999b). After EMT, tumor cells lose cell-cell contact and become more invasive because of the increased migration ability (Akhurst and Balmain, 1999; Cui et al., 1996; Ellenrieder et al., 2001; Portella et al., 1998).

TGFβ signals are transduced into nuclei by intracellular mediator SMADs. Based on their functions in the TGFβ signaling pathway, SMADs are divided into three subtypes, including receptor activated SMADs (MADHs – Human Gene Nomenclature Database), SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8; inhibitory SMADs, SMAD6 and SMAD7; and a common SMAD, SMAD4 (reviewed in Heldin et al., 1997; Massague, 1998). *SMAD4* was cloned as a tumor suppressor gene, deleted in pancreatic cancer (*DPC4*) (Hahn et al., 1996a). Loss-of-function mutations of *SMAD4* are frequently detected in pancreatic cancer, colon cancer, and gastric polyposis and adenocarcinomas (Friedl et al., 1999; Hahn et al., 1996b; Howe et al., 1998; Tamura et al., 1996).

Targeted disruption of Smad (Madh – Mouse Genome Informatics) genes in mice has revealed multiple essential roles of these proteins in mammalian development (reviewed by Weinstein et al., 2000). It was shown that loss of *Smad4* results in lethality at embryonic (E) days 6-7 because of impaired extra-embryonic membrane formation and decreased epiblast proliferation (Sirard et al., 1998; Yang et al., 1998). To overcome the early lethality and to study functions of Smad4 during later stages, especially during mammary gland development and neoplasia, we performed a mammary epithelium specific knockout of *Smad4* using the Cre-loxP system. Our data showed that the disruption of *Smad4* in mammary epithelium overall does not disrupt normal development of mammary glands. However, it results in the formation of squamous cell carcinoma and mammary abscesses primarily caused by transdifferentiation of mammary epithelium to squamous epithelium caused by the loss of TGFβ responsiveness. These observations uncover a role of Smad4 in cell fate maintenance during mammary gland development and mammary cycle progression.

Materials and methods

Mice and mating

The $Smad4^{Co/Co}$WAP-Cre and $Smad4^{Co/Co}$MMTV-Cre mice were generated by crossing the $Smad4^{Co/+}$ mice (Yang et al., 2002a) with WAP-Cre or MMTV-Cre transgenic mice (Wagner et al., 1997), respectively. The $Smad4^{Co/Co}$WAP-Cre and $Smad4^{Co/Co}$MMTV-Cre female mice were kept with males for continuous mating and the number of pregnancy was recorded. After this mating, the mutant mice were on the average of 25% of 129, 25% of Black Swiss and 50% of FVB. Genotyping and detection of Cre-mediated recombination were as described (Wagner et al., 1997; Yang et al., 2002a). When sacrificed, one of the fourth glands was used for whole-mount preparation and the others used for DNA, RNA and/or histology analysis. The protocol for animal studies was approved by the 'Animal Care and Use Committee' of the NIDDK.

Northern blots, TUNEL assay and whole-mount staining of mammary glands

RNA was isolated from the mammary glands of female mice during different developmental stages using RNA Tet-60 based on the protocol recommended by the manufacturer (Tel-Test 'B, Friendswood, TX). About 20 μg of total RNA from each sample was loaded on 1% agarose gel and transferred to a Gene-Screen filter after electrophoreses. TUNEL assay was carried as recommended by the manufacturer (Intergen Company, Purchase, NY). Whole-mount staining of mammary glands was carried out as described (Robinson and Hennighausen, 1997).

Histology, immunohistochemical staining and western blotting

For histology, tissues were fixed in 10% formalin, blocked in paraffin wax, sectioned, stained with Hematoxylin and Eosin, and examined by light microscopy. Detection of primary antibodies was performed using the ZYMED Histomouse^{TM} SP Kit according to the manufacturer's instructions. Western analysis was performed using standard procedures. Cyclin D1, ErbB2 and Smad4 antibodies were purchased from Santa Cruz Biotechnology. Antibodies for K14, K10 and BrdU were purchased from Covance; and β-catenin and E-cadherin were from BD Transduction Laboratories.

Generation of cell lines and TGFβ treatment

Generation and maintaining of cell lines from mammary tissues, tumors and abscesses were as described (Brodie et al., 2001). Cells (2×10^6) were plated into 10 cm plate for morphogenic transformation analysis and 1×10^5 cells were seeded on 22×22 mm glass slides for immunofluorescence analysis. The next day, cells were treated with 2 ng/ml of TGFβ1 for various time points as indicated in Fig. 7 before being harvested for analysis.

Results

Smad4 expression in adult tissues

Northern blot analysis demonstrated the presence of *Smad4* in all examined tissues/organs of adult mice (Fig. 1A). Smad4 expression in mammary tissue was detected in the virgin tissue, during pregnancy, lactation and involution (Fig. 1B). We next performed immunohistochemical staining on mammary glands of these developmental stages using an antibody to Smad4, and found that Smad4 was detected at a nearly ubiquitous pattern (Fig. 1C-F). This expression pattern suggests that Smad4 may play an important role in mammary gland development.

Targeted disruption of Smad4 in mouse mammary epithelium

To study the role of Smad4 during mammary gland development and neoplasia, we crossed a mouse strain that carries a Smad4 conditional allele ($Smad4^{Co}$) (Yang et al., 2002a) with *WAP-Cre* or *MMTV-Cre* transgenic mice (Wagner *et al.*, 1997). The $Smad4^{Co/+}$*WAP-Cre* or $Smad4^{Co/+}$*MMTV-Cre* mice were further crossed with $Smad4^{Co/+}$ mice to generate $Smad4^{Co/Co}$*WAP-Cre* or $Smad4^{Co/Co}$*MMTV-Cre* mice. Our analysis indicated that both strains showed very similar phenotypes in the mammary gland development and neoplasia. However, $Smad4^{Co/Co}$*MMTV-Cre* mice also exhibited abnormalities in skin because of *MMTV-Cre* expression in keratinocytes (W.Q. and C.D., unpublished). Therefore, the

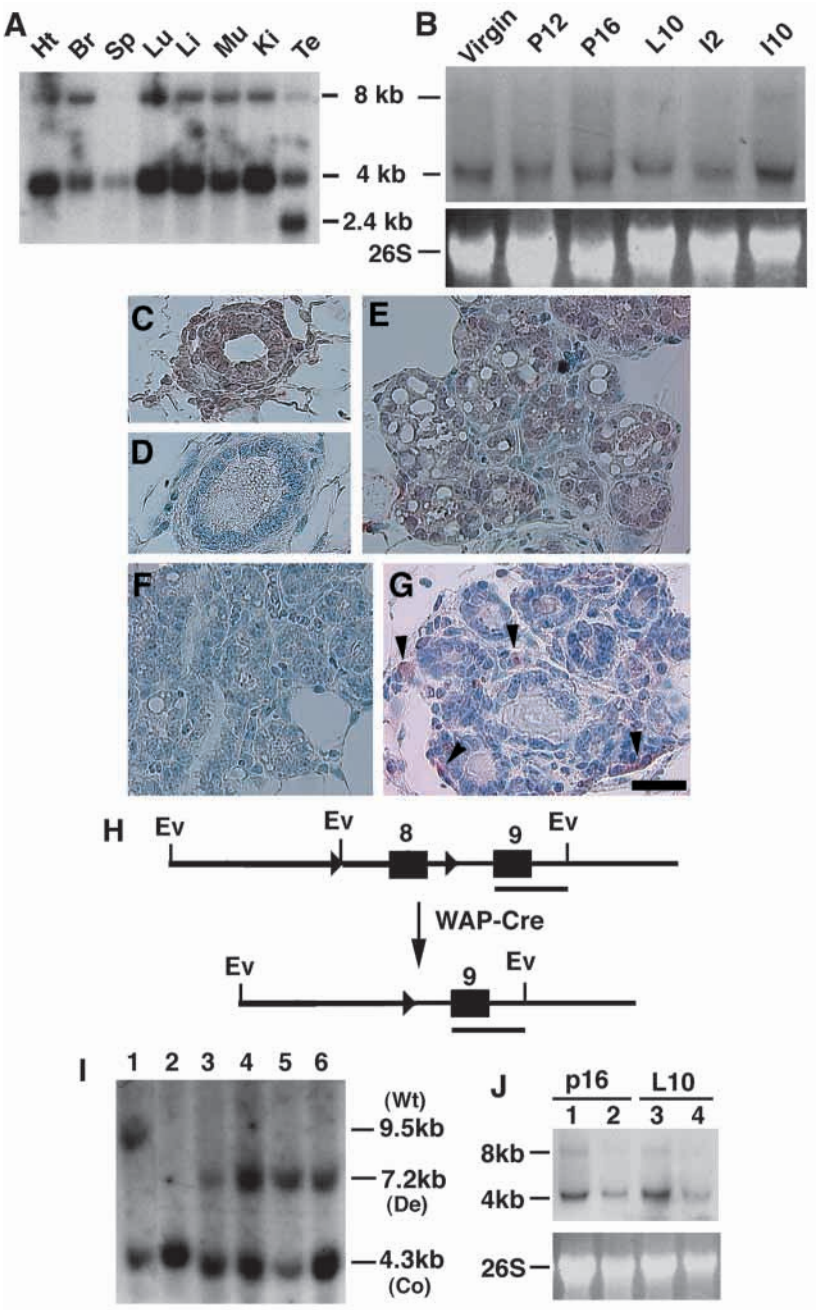

Fig. 1. Expression of *Smad4* in adult mouse tissues and conditional knockout *Smad4* in mammary glands. (A,B) Northern blot analysis of *Smad4* expression in adult tissues. Filters bearing poly A^+ RNA (A, purchased from Clontech) and total RNA (B) were probed with a probe specific to *Smad4* cDNA. Ht, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; Mu, skeletal Muscle; Ki, kidney; Te, testis. (C-G) Immunohistochemical staining using an antibody to Smad4. Primary antibody was not used in sections shown in (D,F) to serve as controls. (C,D) Sections from a wild-type virgin gland. (E,F) Sections from a P14 wild-type gland. (G) A section from an I10 mutant gland, which shows very few cells that are still positive for Smad4 (arrowheads). (H) A *Smad4* conditional (Co) and deletion (De) alleles. Deletion of *Smad4* exon 8 in the mammary gland is achieved by crossing the *Smad4* conditional mice with *WAP-Cre* transgenic mice. (I) Southern blot showing Cre mediated recombination. DNAs were digested with *Eco*RV and probed with a 2.2 kb *Hpa*I-*Eco*RV fragment (black bar). Samples are from mammary glands of $Smad4^{Co/+}$ mice (the first lane) and $Smad4^{Co/Co}$*WAP-Cre* (lane 2-6) mice at different stages, including virgin, P16.5, L2, L10 and I10. 9.5 kb, 7.2 kb and 4.3 kb fragments are wild-type (Wt), deleted (De) and conditional (Co) alleles, respectively. (J) Northern blot analysis of RNA isolated from p16 and L10 wild-type (lanes 1 and 3) and mutant (lanes 2 and 4) mice. At least three mutant and control mice at each point were examined. The intensities of bands were measured using software, IP-lab and normalized with loading controls. % expression of mutant relative to control=average intensity of mutant/average intensity of control×100/100. Scale bar: 60 μm for C-G.

majority of data presented here are based on studies using $Smad4^{Co/Co}$*WAP-Cre* mice.

Cre mediated excision of exon 8 in different tissues isolated from $Smad4^{Co/Co}$*WAP-Cre* mouse was evaluated by PCR and Southern blot analyses. PCR analysis on skin, heart, lung, liver, thymus, ovary, pancreas, brain, skeletal muscle, kidney, spleen and mammary gland demonstrated that the excision occurred exclusively in mammary tissues (not shown). Southern blot analysis of mammary tissue of $Smad4^{Co/Co}$*WAP-Cre* mice (n>15) at several stages of development, including virgin, pregnancy (P) day 16.5, lactation (L) day 2 and 10, and involution (I) day 10, revealed extensive Cre-mediated recombination, which peaked to ~60% at L10 (Fig. 1I). The presence of an unrecombined allele reflects to a large extent the presence of mammary stroma, which is not targeted by the *WAP-Cre* transgene. We detected no recombination in virgin glands, as WAP-Cre is not expressed at this stage. Because mammary tissue consists not only of secretory epithelial cells but also of fat and stroma cells that do not express the *WAP-Cre* transgene, the amount of epithelial cells that undergo recombination may be higher. Indeed, northern blot analysis demonstrated that *Smad4* transcripts were reduced to about 10 and 20% of original levels in mammary tissue from P16.5 (n=5) and L10 (n=5) $Smad4^{Co/Co}$*WAP-Cre* mice, respectively (Fig. 1J). Our immunohistochemical staining using an antibody to Smad4 on mutant mammary glands (n=5) isolated from different developmental stages confirmed that majority of mammary epithelial cells (over 90%) were negative for the staining (Fig. 1G and not shown). Collectively, these observations indicate that *WAP-Cre* has achieved high efficiencies in deleting Smad4 in mammary epithelium.

Absence of Smad4 does not compromise mammary gland development and function

Mammary tissues of $Smad4^{Co/Co}$*WAP-Cre* and $Smad4^{Co/Co}$*MMTV-Cre* mice isolated from different developmental stages, including virgin, P11.5, P14.5, P16.5, L2, L10, I2 and I10, were carefully examined using whole-mount staining and histological sections under microscope. Normal mammary development was observed in all mutant mice examined (n>30) during the first two to three pregnancies

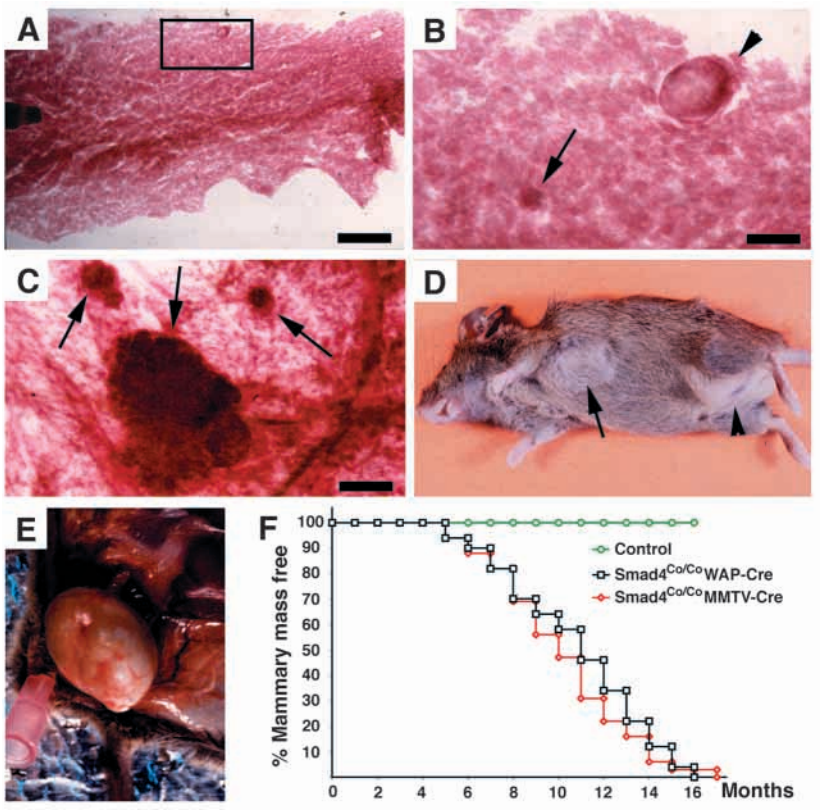

Fig. 2. Mammary tumor mass formation in *Smad4*$^{Co/Co}$*WAP-Cre* and *Smad4*$^{Co/Co}$*MMTV-Cre* mice. (A) Morphologically denser areas detected in the fourth mammary gland of a P16.5 *Smad4*$^{Co/Co}$*WAP-Cre* mouse. This female is 6 months of age and went through more than four pregnancies. (B) An enlarged view of the boxed area shown in A. Arrowhead and arrow indicate areas with different morphology, respectively. (C) Multiple tumor masses (arrows) in a third gland of a one-year-old *Smad4*$^{Co/Co}$*WAP-Cre* mouse. (D,E) An 8-month-old *Smad4*$^{Co/Co}$*WAP-Cre* mouse developed multiple mammary abscesses, two of which are indicated by an arrow and an arrowhead. The area indicated by the arrowhead is shown in E. (F) A curve showing percent of mammary mass *Smad4*$^{Co/Co}$*WAP-Cre* (*n*=36), *Smad4*$^{Co/Co}$*MMTV-Cre* (*n*=34) and control mice. The controls include *Smad4*$^{Co/+}$*WAP-Cre*, *WAP-Cre* and *Smad4*$^{Co/Co}$ mice. About 50 controls younger than 1 year and 20 between 1 and 1.5 years of age were followed and none developed mammary tumor and/or abscess. Scale bar: 3.5 mm in A; 0.87 mm in B,C.

and dams were able to nurse their litters (data not shown). As *Smad4* is expressed in mammary epithelium during all stages of development and Cre-mediated recombination occurred in majority of cells, we conclude that the absence of *Smad4* does not interfere with normal development of the mammary gland.

Mammary tumor and abscess formation in the absence of Smad4

Lack of TGFβ signals results in mammary tumor formation (reviewed by Derynck et al., 2001; Wakefield et al., 2001). We therefore continuously bred Smad4$^{Co/Co}$WAP-Cre dams and monitored for the appearance of mammary tumors. Whole-mount staining revealed dense areas in mammary glands of mutant mice after multiple pregnancies (Fig. 2A-C). Starting at 5 months of age, some Smad4$^{Co/Co}$WAP-Cre mice developed visible tumor masses in their mammary glands (Fig. 2D,F). By 12 months, more than 60% of Smad4$^{Co/Co}$WAP-Cre mice developed tumor masses. The majority of mutant mice contained multiple tumor masses of varying sizes per gland (Fig. 2C,D). By 16 months of age, all Smad4$^{Co/Co}$WAP-Cre mice had developed mammary tumor masses (Fig. 2F). Examination of the majority of these mice at autopsy revealed no sign of tumor metastasis. Our examination on Smad4$^{Co/Co}$MMTV-Cre mice under same mating condition obtained a very similar result (Fig. 2F). Because MMTV-Cre is also expressed in virgin mice and causes gene deletion to lesser extent than it does during pregnancy (not shown), we next studied virgin Smad4$^{Co/Co}$MMTV-Cre (*n*=20) mice and found that they all exhibited similar phenotypes with reduced multiplicity compared with continuously mated group (data not shown). Considering that the MMTV-Cre is expressed less efficiently in virgin mice, this observation suggests that pregnancy related hormones, such as estrogen, do not have an obvious influence on squamous metaplasia and tumorigenesis.

A close examination on the initiating tumors indicated that most of them were solid masses when they were small (<1.5 mm in diameter) (Fig. 2C). However, when they became bigger, the majority gradually turned into cystic abscesses (Fig. 2E), although smaller abscesses (<1 mm) were sometimes observed (arrowhead, Fig. 2B). We next performed histological analysis on *Smad4*$^{Co/Co}$*WAP-Cre* mammary glands to study how the mammary abscess initiated. Sections crossing dense areas revealed alveolar hyperplasia (Fig. 3B) and solid cell masses with undifferentiated alveolar structures (Fig. 3C), suggesting that loss of Smad4 induced abnormal increased proliferation of epithelial cells. In most cases, these cells could not maintain their undifferentiated state and underwent squamous metaplasia. Consequently, tumor masses with diameters about 0.2 cm and bigger often exhibit cancer-pearl-like keratinization and onion-skin-like structures, which are commonly observed in squamous cell carcinomas (Fig. 3E,F). Abscesses with varying sizes that are filled with keratin were found in most mutant glands (arrows in Fig. 3F,G). Our analysis on over 50 tumor-bearing mice indicated that majority of them (>90%) contained both squamous cell carcinoma and mammary abscesses. Tumors with other distinct histopathologies (Fig. 3H,I) were also found at much lower frequencies (<5%). Metaplasia in situ was also occasionally observed (Fig. 3D). This may represent a type of transdifferentiation through which mammary epithelial cells at the hyperplasia stage are directly converted into keratinocytes.

Mammary abscesses exhibit features resembling skin epidermis

The observation that the majority of mammary tumors and abscesses exhibited extensive keratinization prompted us to compare their structures with those of skin. Skin of Smad4$^{Co/Co}$WAP-Cre mice appeared normal, as WAP-Cre is

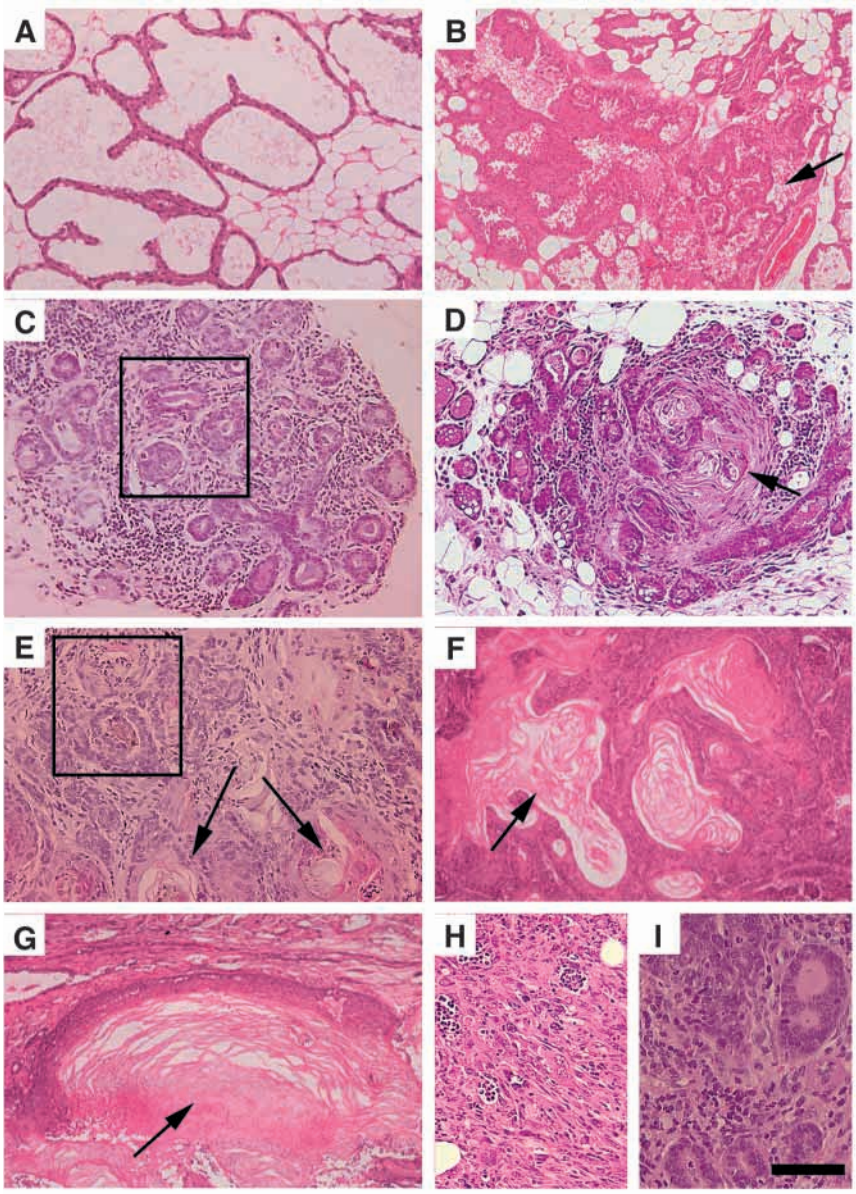

Fig. 3. Histology of mammary tumor in *Smad4$^{Co/Co}$WAP-Cre* mice. (A,B) Normal (A) and hyperplasia (B) area of a L10 gland from a 6-month old mouse. (C) A mammary tumor mass at early stages showing undifferentiated alveolar structures. (D) In situ lesions from a 12-month-old mouse. The arrow indicates the center of initiating abscess. (E-G) Squamous cell carcinoma (E,F) and an abscess (G), showing cancer-pearl-like keratinization and onion-skin-like structures. Notice the increasing sizes of abscesses (arrows). (The boxed areas in C and E were further analyzed in Fig. 6C-F,G-J using molecular markers.) (H,I) The mutant mice also developed other types of tumors at much lower frequencies, such as carcinosarcoma (H) and adenocarcinoma (I). Scale bars: 120 μm in A,C; 240 μm in B,F,G; 136 μm in D; 90 μm in E; 80 μm in H; 60 μm in I.

not expressed in skin (not shown). We used the skin covering the ventral side of the third mammary gland of Smad4$^{Co/Co}$WAP-Cre mice as control (Fig. 4A). The wall of abscesses was composed of multiple stratified squamous epithelial structures that are very similar to those seen in skin (Fig. 4A,B). BrdU-positive cells were detected in the basal layer of both skin (Fig. 4C) and the outside walls of the mammary abscess (Fig. 4D). K14 (Fig. 4E,F) and K10 (Fig. 4G,H) were similarly evident in both normal skin and mammary abscesses. Keratin was accumulated in the center of mammary abscesses (Fig. 4B,D,F,H), whereas in skin, it was present in the outside layer (Fig. 4A,C,E,G) and was sloughed off during skin grows. These observations indicated that lack of Smad4 in mammary epithelium recaptures a process resembling the formation of skin epidermis. The keratin secreted by differentiating keratinocytes blocks the mammary ducts and eventually results in the formation of mammary abscesses.

Transdifferentiation of mammary epithelial cells to keratinocytes occurs during early stages of neoplasia

Although keratin pearls are sometimes seen in late-stage mammary tumors, squamous metaplasia leading to the formation of extensive epidermis like structures in mammary tissues is rarely observed in other transgenic mice (reviewed in Cardiff et al., 2000). To investigate when and how the epidermalization occur, we studied the early stages of tumorigenesis in *Smad4$^{Co/Co}$WAP-Cre* mice. BrdU labeling revealed increased proliferation of epithelial cells in hyperplasic areas (Fig. 5B). However, when pearl-like structures started to form, BrdU labeling was found at the edge of these structures (Fig. 5C,D), while cells in the center were enlarged and underwent transdifferentiation (arrow, Fig. 5C). Immunohistochemical staining for K14, a marker for basal layer keratinocytes of skin, confirmed that these cells had undergone fate changes from epithelial cells to keratinocytes (Fig. 5E,G). Thus, the BrdU-positive cells in the periphery were responsible for the continuous proliferation leading to increased volume of the pearl-like structures, while the cells in the center were responsible for the continuous keratinization (Fig. 5F,G). These observations provide a basis for the neoplasia initiation and continued growth in Smad4-null mammary tissue. To confirm that the K14 expressing cells had undergone Cre-mediated recombination of the Smad4 gene, we performed microdissection of these cells using laser-capture microscopy (Fig. 5H-J). PCR analysis on the dissected cells demonstrated that they had undergone Cre-mediated recombination (Fig. 5K,L). Together, these data indicated that loss of Smad4 promotes proliferation in early stages of neoplasia; however, at later stages, it causes epithelial cells to undergo transdifferentiation into keratinocytes, leading to the formation of the epidermis like structures.

Accumulation of β-catenin was associated with early signs of squamous metaplasia

Smad4-null mammary tissue and mammary abscesses were

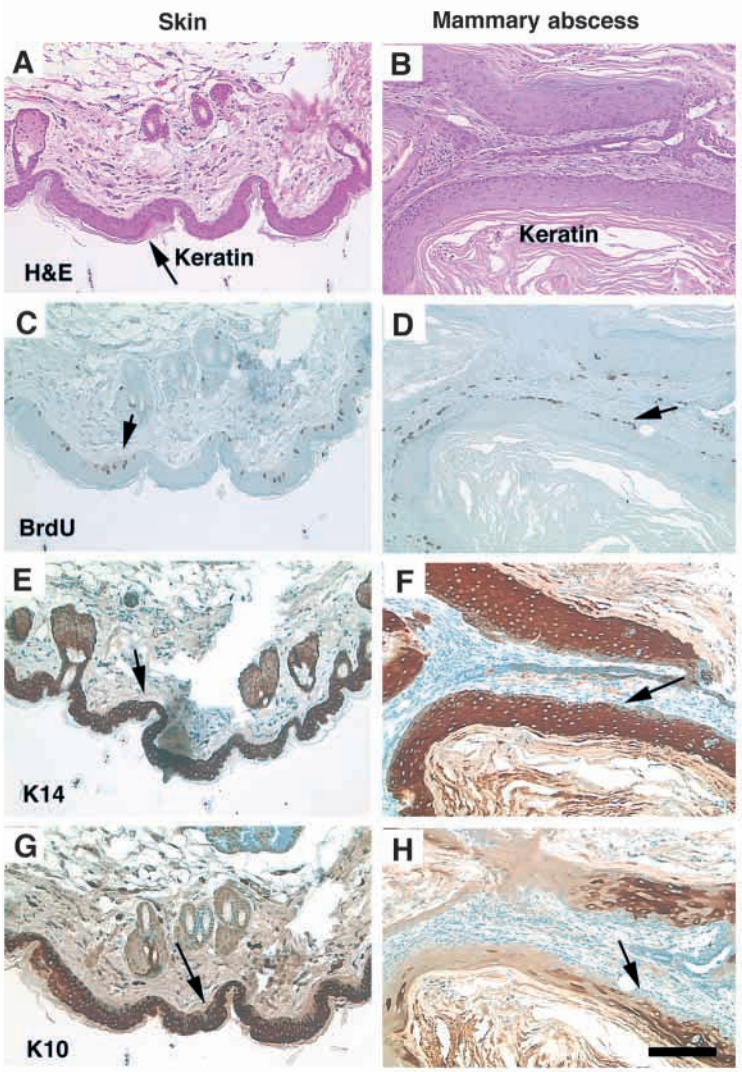

Fig. 4. Conditional knockout of Smad4 results in epidermis formation in mammary glands. (A,C,E,G) Skin samples; (B,D,F,H) abscesses found in the fourth gland from a 7.5-month-old *Smad4*$^{Co/Co}$*WAP-Cre* mouse. Markers used for analysis were as indicated. At least 12 abscesses were studied and similar features were observed. Scale bar: 125 μm.

further analyzed to uncover molecular mechanism(s) underlying the squamous metaplasia. TUNEL assay revealed no apparent differences between mutant and control glands when examined at stages prior to tumor initiation (not shown). We also detected no changes in the expression of number of known tumor suppressor genes and oncogenes, including p63, Myc, β-catenin, E-cadherin, cyclin D1, p21 and Erbb2 in normal mutant and control glands using in situ hybridization or immunohistochemical staining (not shown), suggesting that the loss of Smad4 does not directly affect expression of these genes at this stage (prior to tumor initiation). We next analyzed gene expression in mammary abscesses by western blot and found significantly high levels of K14, β-catenin and E-cadherin (Fig. 6A). The high levels of K14 are consistent with the increased expression of K14 detected by immunohistochemical staining (Fig. 5). We then analyzed expression patterns of β-catenin and E-cadherin in abscesses (n>12) of varying sizes by immunohistochemical staining. Our data indicated that β-catenin was expressed in all abscesses at high levels, irrespective to their sizes and developmental stages (arrows, Fig. 6B), while E-cadherin was often present at low levels at initiating abscesses (not shown and see below). To further address the roles of these proteins in squamous metaplasia, we compared expression of β-catenin, E-cadherin and K14 in in situ lesions during three arbitrary phases associated with abscess initiation and development using immunofluorescent staining. This includes phase I, prior to a visible sign of squamous metaplasia; phase II, initiating squamous metaplasia; and phase III, during and immediately after squamous metaplasia. We showed that K14 is expressed at a low level in phase I lesions (Fig. 6C,D; arrowhead in 6G,H), and at high levels in phase II (arrow in Fig. 6G,H) and phase III (Fig. 6K,L) lesions. Next, we checked β-catenin expression and found that it was expressed at higher levels in the phase I lesions (Fig. 6E; arrowhead in 6I) than in wild-type control epithelial cells (not shown), although some variations were observed. While this observation suggests that these lesions may be at different degrees of squamous metaplasia, we showed that β-catenin expression became more intense in phases II (arrow in Fig. 6I) and III (Fig. 6M) lesions. After superimposing (Fig. 6F,J,N), it is clear that upregulation of β-catenin precedes that of K14 (compare arrow and arrowhead in Fig. 6G-J). However, our analysis revealed no significant expression of E-cadherin at initiating abscesses (Fig. 6Q). Thus, our data reveal that the strong expression of β-catenin is associated with the first sign of transdifferentiation and the expression is also tightly associated with the progression of metaplasia. These observations implicate β-catenin as a top candidate, the dysregulation of which is responsible for the abnormalities observed in the Smad4-null mammary glands.

Smad4$^{-/-}$ cells lost TGFβ-mediated β-catenin degradation and failed to undergo TGFβ-induced EMT

Alterations of β-catenin has been shown to cause multiple types of human cancers (Akiyama, 2000; Karayiannakis et al., 2001; Polakis, 2001). However, the relationship between β-catenin, which serves as a major component of Wnt signaling pathway, and TGFβ signals is not clear. To address this, we sequenced RT-PCR products from six mammary squamous carcinoma and abscesses using primers that amplify 390 bp cDNA fragment, including exon 2 of β-catenin, which is a hot spot for mutations. We failed to detect any mutations in the gene, suggesting that the increased β-catenin was not caused by mutations that stabilize the protein. Next, we investigated whether β-catenin levels could be regulated by TGFβ/Smad4 signals in cultured cells. For this experiment, Smad4-null and wild-type cell lines were subjected to TGFβ1 treatment. Western blot analysis revealed that TGFβ1 treatment significantly decreased β-catenin in three *Smad4*$^{+/+}$ (wild-type) cells examined, including one cell line derived from a wild-type mammary gland and two cell lines derived from mammary tumors of *MMTV-ras* transgenic (Sinn et al., 1987) and *Brca1*

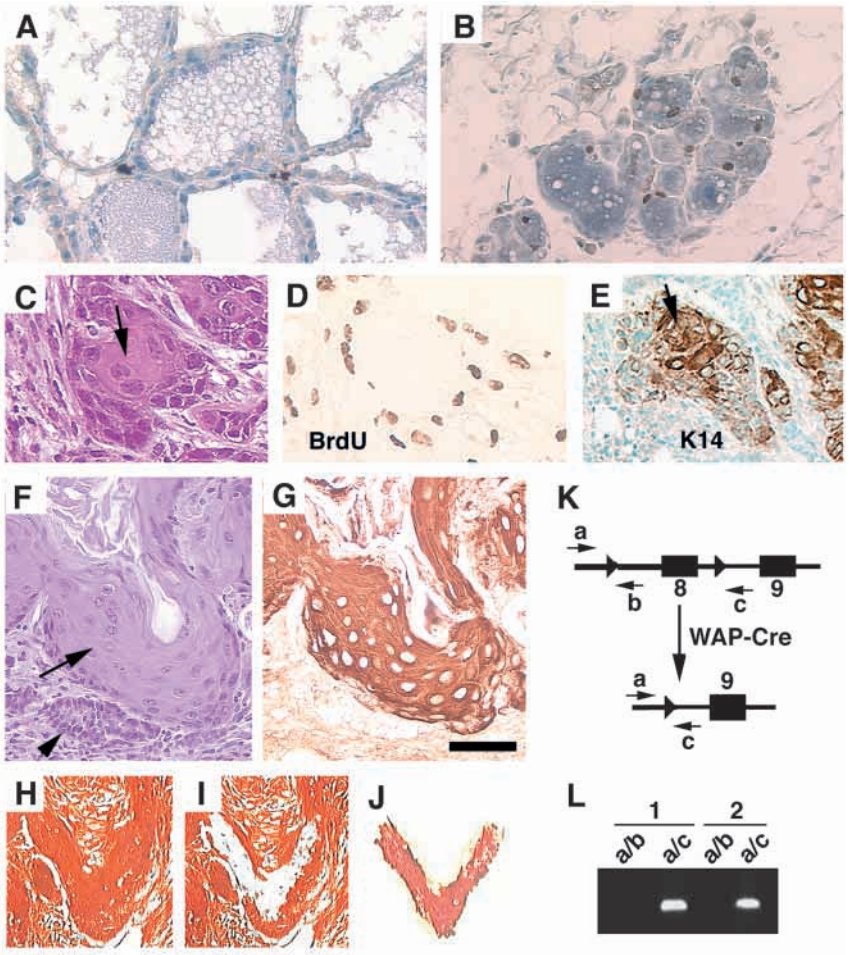

Fig. 5. Transdifferentiation from mammary epithelial cells to keratinocytes due to the loss of Smad4. (A,B) BrdU labeling of normal (A) and hyperplasia (B) area of a L10 gland. We have counted $BrdU^+$ cells in 12 equivalent normal (n=6) and hyperplasia (n=6) areas. Average percentages of $BrdU^+$ cells are 0.85±0.63 and 8.5±1 in normal and hyperplasia, with the P<0.01 by Student's t-test. (C-E) Images of early stages of transdifferentiation revealed by H&E (C), BrdU (D) and K14 (E) immunohistochemical staining. Arrows indicate the center of the lesion. (F,G) Images of an abscess at later stages detected by H&E (F) and K14 (G) immunohistochemical staining. Arrow indicates to the wall of the abscess, which is K14 positive, and arrowhead indicates an alveoli, which is K14 negative. (H-L) Micro-dissection (H-J) and PCR genotype (K,L) of dissected abscesses. (H) Prior to dissection; (I) after dissection; and (J) the dissected sample. (K) Structure of floxed allele. (L) Two independent samples (1 and 2) collected by micro-dissection, were analyzed and both showed that the abscess is caused by the Cre-mediated deletion of *Smad4*. Primers a/c amplify about 500 bp from the recombined allele. Primers a/c amplify about 450 bp from conditional allele, which is absent in these samples. The sequences of the primers are: a, 5′-GACCCAAACGTCACCTTCAG-3′; b, 5′-GGGCAGCGTAGCATATAAGA-3′; and c, 5′-AAGAGCCACAGGTCAAGCAG-3′. All samples were from a 7-month-old $Smad4^{Co/Co}$*WAP-Cre* mouse. Scale bar: 180 μm in A,B; 35 μm in C-E; 60 μm in F,G.

mutant (Xu et al., 1999) mice, respectively (Fig. 7A). By contrast, our study revealed no changes in β-catenin levels upon the treatment with TGFβ1 in two *Smad4*-null cell lines independently derived from mammary absences, as assayed by both western blot (Fig. 7B) and immunofluorescence (Fig. 7C). These observations suggest that TGFβ1 treatment results in β-catenin degradation, which is mediated by Smad4.

TGFβ has been known as a potent factor that promotes EMT of cultured mammary epithelial cells (Piek et al., 1999a). The existence of Smad4-null epithelial cells provides an excellent opportunity to study the role of Smad4 in this process. Thus, we treated the cells with TGFβ1 at multiple time points, ranging from 0, 2, 4, 6, 12, 24 and 48 hours. We found that both Smad4-null cells lost the TGFβ responsiveness and showed no signs of EMT (Fig. 7C). By contrast, EMT was readily observed in four $Smad4^{+/+}$ cell lines tested (Fig. 7D and not shown), indicating that Smad4 is essential for TGFβ induced cell fate transformation.

We have also followed progression of EMT and dynamic changes of β-catenin upon TGFβ treatment in wild-type cells. Our result indicated that the morphological transformation became apparent 12 hours after TGFβ treatment and the EMT was more obvious at later time points (Fig. 7D). A slightly lower level of β-catenin in the TGFβ treated than untreated cells was observed at 6 hours and a further reduction of β-catenin occurred at 12 hours and later points (Fig. 7D). Thus, the downregulation of β-catenin correlates with the onset of cell fate transformation, suggesting that β-catenin may play an active role in TGFβ-induced EMT.

Discussion

The ectopic expression of TGFβs in the mammary epithelium of transgenic mice results in abrogated development and loss of differentiation (reviewed by Barcellos-Hoff and Ewan, 2000). TGFβs, their receptors and Smad4 are expressed during all stages of mammary gland development (Chakravarthy et al., 1999; Daniel et al., 1996; Wakefield et al., 2001; Walker, 2000), suggesting that TGFβ signals could function through Smad4 during mammary gland development. To test this hypothesis directly, we inactivated the *Smad4* gene in mouse mammary epithelium and studied roles of TGFβ/Smad4 signals in pregnancy-mediated development and tumorigenesis. We demonstrate that the loss of Smad4 does not cause obvious abnormalities in the mammary gland up to the first three pregnancies. However, it resulted in the formation of mammary neoplasia, which was characterized by squamous cell carcinoma and mammary abscesses. Furthermore, we correlated the formation of squamous metaplasia with an increase of β-catenin. Although β-catenin protein levels were reduced by TGFβ1 in cultured mammary epithelial cells, no such reduction was observed in the absence of Smad4. These observations uncover an essential role for Smad4 signals in cell fate maintenance during mammary gland development.

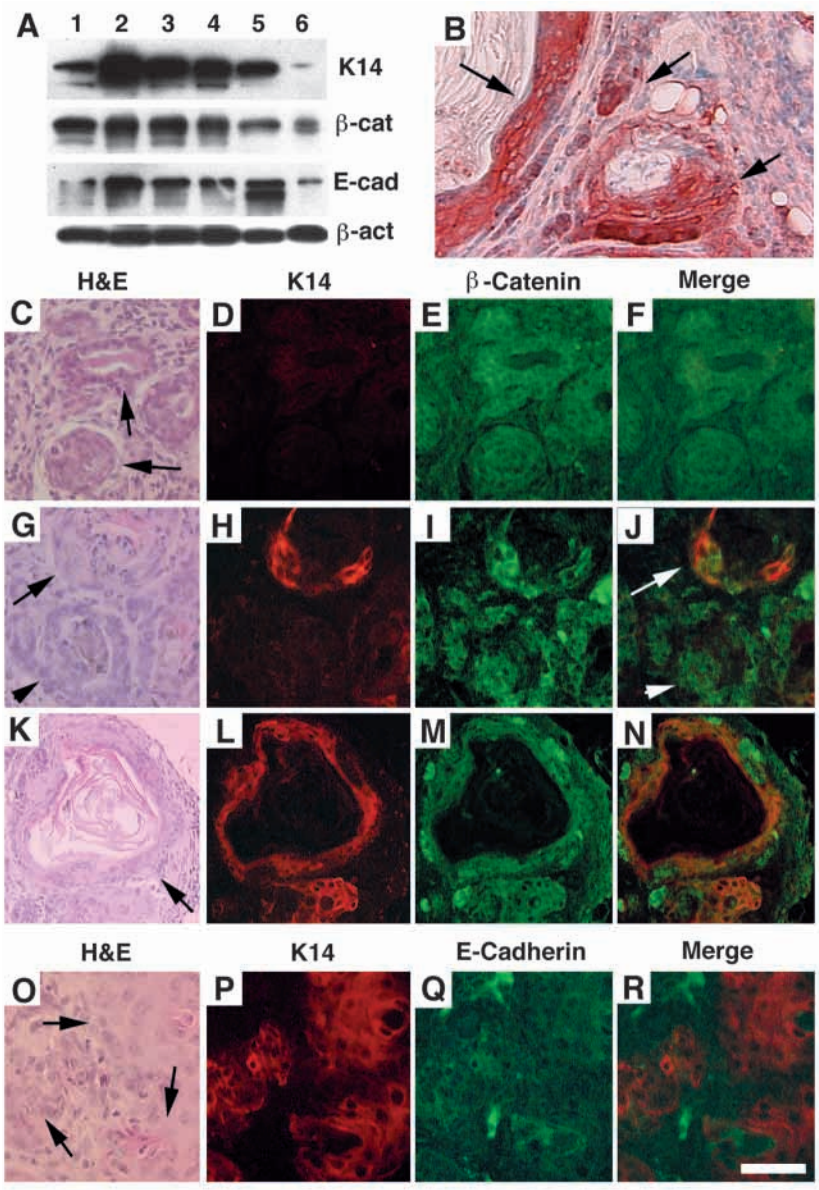

Fig. 6. β-catenin overexpression at the earliest stage of transdifferentiation. (A) Western blot showing expression of K14, β-catenin and E-cadherin in mammary abscesses (lanes 1-5) and adjacent normal gland (lane 6). (B) All abscesses examined, irrespective of their sizes (arrows), are positive for β-catenin as demonstrated by this section from a 1-year-old *Smad4*$^{Co/Co}$*WAP-Cre* mouse. (C-R) Analyzing squamous metaplasia at three distinct phases as described in the text: phase I (C-F, and arrowhead in G-J); phase II (arrow in G-J); and phase III (K-R). C,G are amplified from Fig. 3C,E, respectively. Antibodies used are as indicated. Arrows in C indicate undifferentiated alveolar structures. The arrow and arrowhead in G indicate the differentiated and undifferentiated alveolar structures, respectively. Notice that cells prior to differentiation (arrowhead) express only β-catenin but not K-14, while the differentiating cells (arrow) express both. The arrow in K indicates a small, but well formed abscess that expresses both genes. The arrows in O indicate initiating metaplasia, which express K14 at high levels but E-cadherin at significantly lower levels. Scale bars: 140 μm in K-N; 70 μm in B-J,O-R.

Although Smad4-null embryos die at E6.5 and display defects in cell proliferation (Sirard et al., 1998; Yang et al., 1998), the loss of Smad4 in mammary epithelium does not disrupt mammary development during the first a few pregnancies. It is possible that the TGFβ/Smad4 signals do not contribute to the proliferation and differentiation of the mammary epithelium, or that TGFβ/Smad4 signals can be compensated for by the presence of other factors. As Smad4 serves as a central mediator for TGFβ signals (reviewed by Heldin et al., 1997; Massague, 1998), we favor the second possibility. Moreover, recent studies with cultured cells revealed that TGFβ signals can be relayed through a number of additional mediators, such as MAPK, EGF and HGF (Engel et al., 1999; Hocevar et al., 1999; Kretzschmar et al., 1999; Mulder, 2000). Thus, in the absence of Smad4, TGFβ signals possibly activate some of their downstream targets in *Smad4*-null mammary epithelium.

The absence of Smad4 in the mammary epithelium reproducibly led to hyperplastic foci with increased cell proliferation. This suggests that the Smad4-mediated TGFβ signaling pathways serve as a negative regulator for mammary epithelial cell proliferation. The long latency in tumor formation may reflect the complexity of growth regulation inherent to the mammary gland. It is likely that TGFβ/Smad4 signals interact with other growth pathways, and that the loss of Smad4 enables the mutant cells to gradually escape growth regulation, which subsequently results in increased proliferation. However, the first burst of proliferation was restricted to the periphery of the developing tumor masses, while the cells in the center underwent transdifferentiation and formed squamous metaplasia, leading to the establishment of squamous cell carcinomas. Notably, the expression of a TGFβ *DNIIR* transgene alone, which specifically blocks the signals of the TGFβ subfamily, only causes hyperplasia and squamous cell carcinoma initiation after carcinogen treatment (Bottinger et al., 1997b). The phenotype observed upon deletion of the Smad4 gene in mammary epithelium was more profound than the overexpression of the TGFβ *DNIIR* transgene, which is consistent with the view that Smad4 is a common mediator, which not only mediates TGFβ subfamily, but also the BMP and activin pathways as well.

Keratin pearls and squamous cell carcinoma are also observed in a number of transgenic mice, including *Pten*$^{+/-}$ (Stambolic et al., 2000), *Apc*$^{474/+}$ (Sasai et al., 2000) and TGFβ *DNIIR* (Bottinger et al., 1997a) mice. However, mammary abscess formation caused by continuous epidermalization is not a feature in these transgenic mice. It is unique that Smad4-associated neoplasia undergoes transdifferentiation to such an extent that it eventually turns into skin-like structures in the mammary glands. Mammary epithelium and epidermis share a common origin as they both derive from the ectoderm. They undergo distinct developmental outcomes as the mammary bud

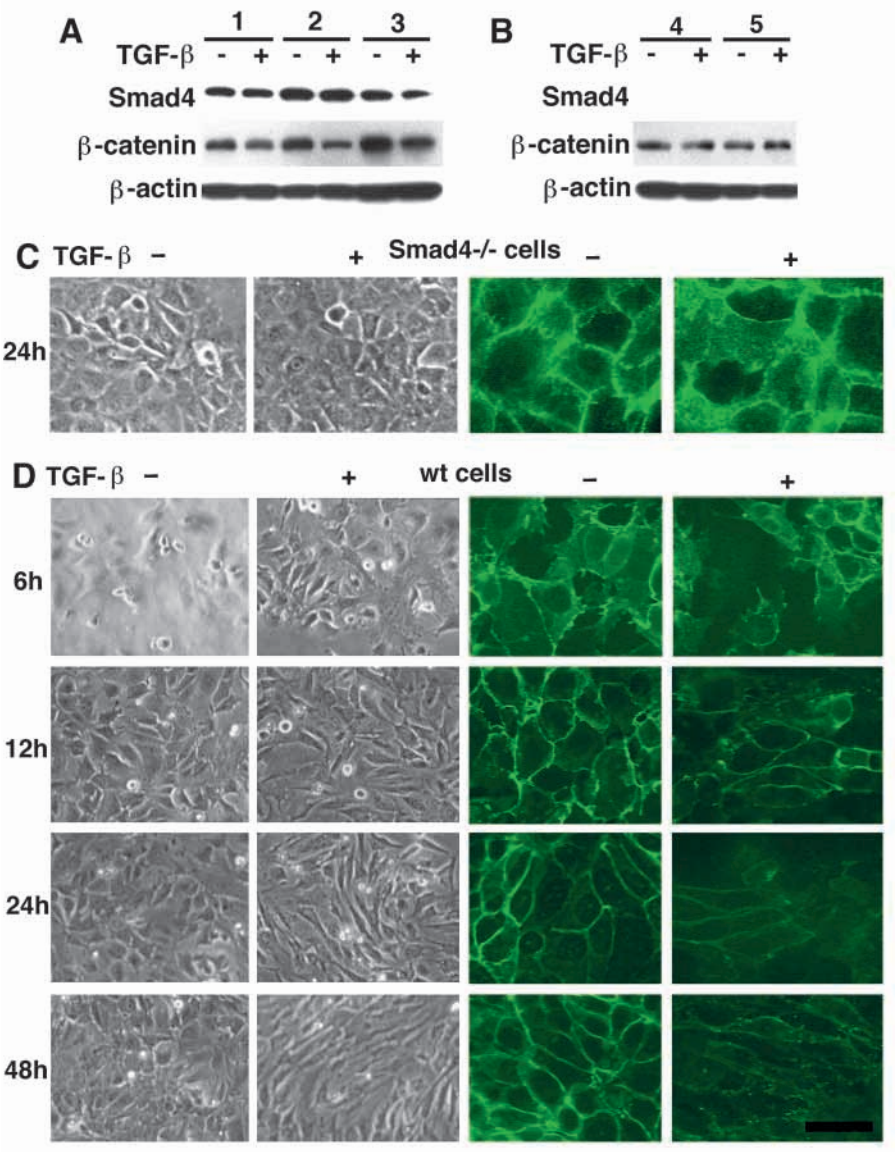

Fig. 7. Loss of TGFβ-mediated β-catenin degradation and TGFβ responsiveness in *Smad4*$^{-/-}$ cells. (A,B) Western blot showing β-catenin in *Smad4*$^{+/+}$ (A) and *Smad4*$^{-/-}$ (B) cells after they were treated with 2 ng/ml of TGFβ for 24 hours. Samples 1 and 2 were mammary tumor cell lines derived from *Brca1* conditional knockout and *MMTV-ras* transgenic mice, respectively. Sample 3 was a cell line derived from a P16 mammary gland from a *Smad4*$^{+/+}$ mouse. Sample 4 and 5 were two *Smad4*$^{-/-}$ cell lines derived independently from mammary abscesses of *Smad4*$^{Co/Co}$*WAP-Cre* mice. Treatment of TGFβ(+) led to a decrease in β-catenin in all *Smad4*$^{+/+}$ cell lines, whereas no changes were detected in both independently derived *Smad4*$^{-/-}$ cells. (C) Loss of Smad4 blocks TGFβ responsiveness as *Smad4*$^{-/-}$ cells did not undergo EMT and failed to decrease β-catenin upon TGFβ treatment. (D) Timecourse response of *Smad4*$^{+/+}$ (wild-type) cells to TGFβ induced EMT and downregulation of β-catenin. Scale bars: 100 μm for phase contract images and 39 μm for immunofluorescent images.

forms ductal branches during embryogenesis (Cardiff et al., 2000). The loss of Smad4 in the mammary epithelium results in a continuous epidermalization, suggesting that TGFβ/Smad4 signals may be normally involved in a process that positively regulates either the transition of the mammary epithelium from embryonic epidermal cells or its maintenance. Because the loss of Smad4 reverses this process, leading to the epidermalization of mammary epithelium, we suggest that Smad4 is required to maintain mammary epithelium and prevent them from undergoing transdifferentiation. Thus, our findings may suggest that the correct dose of TGFβ/Smad4 signals is essential in maintaining normal development of mammary epithelial cells. Both the activation and the inactivation of these signals can cause abnormalities, i.e. induce transdifferentiation of mammary cells to opposite differentiation pathways. This may provide a molecular basis for the long observed dual functions of TGFβ signals (reviewed by Wakefield and Roberts, 2002). Therefore, we propose that TGFβ signals act through Smad4 to inhibit tumor initiation through their ability to inhibit epithelial cell proliferation. When these inhibition signals are absent due to the lack of Smad4, mammary epithelial cells increase proliferation leading to the hyperplasia and tumor initiation. Meanwhile, Smad4 also plays a potent role in determining the fate of the cells. Its absence unavoidably triggers transdifferentiation of mammary epithelial cells. As a net result of losing these functions, Smad4-null mammary epithelial cells undergo both tumorigenesis and continuous transdifferentiation. This results in the conversion of Smad4-null tumor cells into highly differentiated, yet less malignant cells, leading to the mammary abscess formation. It was proposed that TGFβ signals promote tumor metastasis at later stages through inducing EMT (Oft et al., 2002; Oft et al., 1996; Piek et al., 1999a). The mutation we introduced could not directly address this, as it produces a loss-of-function, instead of activation, of TGFβ signals. However, our observation that the absence of Smad4 blocked TGFβ-induced EMT in cultured cells suggests that Smad4 may mediate this action. Although it remains to be confirmed by in vivo studies, the lack of metastasis in all *Smad4*$^{Co/Co}$*WAP-Cre* and *Smad4*$^{Co/Co}$*MMTV-Cre* mice examined (n>50) is consistent with this view.

We showed that the absence of TGFβ/Smad4 signals results in increased levels of β-catenin in vivo, while an activation of these signals leads to a decrease of β-catenin in vitro. We demonstrated that the increase of β-catenin in Smad4-null mammary epithelium occurs prior to and during the transdifferentiation, while in the TGFβ treated cell, the decreased β-catenin occurs at onset of the morphogenic transition. These observations suggest that β-catenin could serve as one of the key molecules that mediate TGFβ/Smad4 signals in determining cell outcome. Recently, it was shown

that constitutive activation of β-catenin in mammary epithelium through the deletion of the N-terminal including the GSK3β phosphorylation sites results in the transdifferentiation of mammary alveolar epithelium into epidermal structures (Miyoshi et al., 2002). However, our analysis failed to detect a mutation in β-catenin, suggesting that a different mechanism, rather than mutations that stabilize the protein, is involved in the increased levels of β-catenin. Of note, alterations of β-catenin were also observed in many human breast cancers without detectable mutations (Candidus et al., 1996; Karayiannakis et al., 2001). Thus, it was proposed that the regulation of β-catenin might occur at transcriptional, translational and/or post-translational levels (Karayiannakis et al., 2001). Our observation that the treatment of TGFβ1 decreased β-catenin levels in wild-type, but not the *Smad4*-null, cells, is consistent with this view. It also suggests a potential interaction between TGFβ/Smad4 and Wnt signals in regulation of β-catenin, although the underlying mechanism is currently unknown and needs further investigation.

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